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CRDEC-TR-129

TOXICITY OF GRAPHITE FLAKES IN SOIL TO EARTHWORMS

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June 1990

U.S. ARMY
ARMAMENT
MUNITIONS
CHEMICAL COMMAND

Aberdeen Proving Ground, Maryland 21010-5423

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1990 June	Final, 88 Jun - 89 Oct

4. TITLE AND SUBTITLE
Toxicity of Graphite Flakes in Soil To Earthworms

5. FUNDING NUMBERS
PR-89AH

6. AUTHOR(S)
Bowser, Lester K., Phillips, Carlton T., and Wentsel, Randall S., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
CDR, CRDEC, ATTN: SMCCR-RSC-C, APG, MD 21010-5423

8. PERFORMING ORGANIZATION REPORT NUMBER
CRDEC-TR-129

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Graphite flakes, a potential candidate for use in various military hardware and equipment, were tested for their toxicity to the earthworm (Eisenia foetida). The graphite flakes were tested at 0% (control), 0.05%, 0.10%, 0.50%, and 1.00% concentrations by weight. These concentrations were used in the study by thoroughly incorporating the graphite flakes into an artificial soil mixture composed of sphagnum peat (10%), kaolinite clay (20%), fine sand (69%), and calcium carbonate (1%). Graphite flakes produced no lethal effects at graphite flake concentrations up to 1.0%. Sublethal effects, measured as weight changes, ranged from net increases of 27.4 to 29.5%. The results indicate that graphite flakes are not toxic to earthworms (E. foetida) at the concentrations tested.

14. SUBJECT TERMS		15. NUMBER OF PAGES
Graphite flakes lethal effects Artificial soil	Earthworms (Eisenia foetida) Sublethal effects	18
		16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

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PREFACE

The work described in this report was authorized under Project No. 89AH. This work was started in June 1988 and completed in October 1989.

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CONTENTS

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TOXICITY OF GRAPHITE FLAKES IN SOIL TO EARTHWORMS

1. INTRODUCTION

Earthworms have been studied as key organisms in the soil community, because of their role in maintaining soil physical characteristics and processes such as aeration water permeability, and breakdown of organic matter. Roberts and Dorough[1] gave a very good review of the importance of earthworms to terrestrial ecosystems and their use in assessing the hazards of chemicals to these nontarget organisms. Earthworms can number up to a quarter million per acre. They increase the fertility of soil by increasing the availability of nutrients. Earthworms are also an important link in the food chain. In another review, Dean-Ross discusses the strengths and weaknesses of experimental methods for testing the toxicity of chemicals to earthworms. Dean-Ross recommended that Lumbricus terrestris and Eisenia foetida (E. foetida) be used as the test species of choice.

The purpose of this study was to develop baseline environmental data on micro 260 graphite flakes (Asbury Graphite Mills, Incorporated, Asbury, NJ). This report presents the results of the toxic effects of the material on the earthworm (E. foetida).

2. METHODS

The method used to determine the toxicity of graphite flakes in soil to the earthworm (Appendix) was adopted from Karnak and Hamelink.[3] E. foetida (purchased from Bert's Bait, Irvine, KY) were the earthworms used in this experiment. The worms were stored in an incubator at 13 °C in peat moss for 2 weeks before use.

The test media consisted of a nonsterile artificial soil and distilled water (Neuhauser et al) 4 The components of the artificial soil follow:

- Finely ground sphagnum peat 10%
- Kaolinite clay 20%
- Fine sand 69%
- Calcium Carbonate 1%

The test medium (Table 1) was prepared by mixing the artificial soil and the proper amount of graphite flakes (micro 260) in a food blender. Next, distilled water was slowly added until a uniform mixture was achieved. The test medium was then placed in a 400 mL beaker. Each beaker was covered with gauze after five worms of comparable weights were added Target concentrations for the graphite flakes were 0. 0.05. 0.10 0 50 and 1 0%.

Table 1. Compounds of Soil Spiked With Graphite Flakes (grams)

Chamber number		Soil wt.	Distilled water	Graphite	% Graphite
Control	1	200.00	70	0.0	0.0
	2	200.00	70	0.0	0.0
	3	200.00	70	0.0	0.0
Level 1					
	1	199.9	70	0.1	0.05
	2	199.9	70	0.1	0 05
	3	199.9	70	0.1	0 05
Level 2					
	1	199.8	70	0.2	0.10
	2	199.8	70	0.2	0.10
	3	199.8	70	0 2	0.10
Level 3					
	1	199.0	70	1.0	0.50
	2	199.0	70	1.0	0 50
	3	199.0	70	1 0	0.50
Level 4					
	1	198 0	70	2.0	1.00
	2	198.0	70	2 0	1 00
	3	198.0	70	2.0	1.00

The experimental design is described below:

TEST CONCENTRATION	REPLICATES
Control	a b c
Level 1	a b c
Level 2	a b c
Level 3	a b c
Level 4	a b c

Five earthworms were weighed as a group and randomly (random number generator) added to a test container. The containers were set randomly into a low temperature incubator at 13 °C + 0.2 °C. After 14 days, the earthworms in each test container were weighed and examined for physical condition.

Bartlett's test for homogenous variance[5] and the Analysis of Covariance (ANCOVA) to test earthworm weight differences[6] were the statistical methods used to evaluate data.

3. RESULTS

The effects of graphite flakes on E. foetida are presented in Table 2. No lethal effects were observed at graphite concentrations up to 1.0%. Sublethal effects, measured as weight changes, ranged from net increases of 27.4 to 29 5%. The statistical evaluation by ANCOVA indicated no significant ($p < 0.05$) differences between treatments (Table 3).

4. DISCUSSION

The use of an artificial soil limits test variability due to changes in soil parameters. The advantages of using the artificial soil mixture are ease of preparation and comparability to other data.

The results of the test indicated that graphite flakes were not toxic to earthworms at the concentrations tested. No lethal or sublethal effects were observed on E. foetida at graphite flake concentrations from 0.05 to 1 0%.

Graphite flakes were much less of a hazard to the environment than brass flakes. Wentsel and Guelta[7] reported a 14-d Lc_{50} of 190 µg/g of brass flakes to the earthworm Lumbricus terrestris.

Table 2. Results of E. foetida Exposed to Graphite Flakes (grams)

Beaker #	# Worms Beg.	Tot. Wt Beg.	# Worms End	Tot. Wt. End	Mean Wt. Beg.	Mean Wt. End	Diff. Wt.
Control							
1	5	1.40	5	1.90			
2	5	1.45	5	2.07	1.37	1.86	0.49
3	5	1.25	5	1.62	±0.10	±0.23	
Level 1							
4	5	1.28	5	1.71			
5	5	1.38	5	1.70	1.32	1.71	0.39
6	5	1.30	5	1.71	±0.05	±0.01	
Level 2							
7	5	1.45	5	1.89			
8	5	1.56	5	1.98	1.48	1.90	0.42
9	5	1.43	5	1.83	±0.07	±0.08	
Level 3							
10	5	1.48	5	1.89			
11	5	1.29	5	1.67	1.34	1.71	0.37
12	5	1.24	5	1.56	±0.13	±0.10	
Level 4							
13	5	1.36	5	1.87			
14	5	1.29	5	1.52	1.35	1.72	0.37
15	5	1.42	5	1.77	±0.07	±0.10	

Table 3. Analysis of Covariance (Adjusted Y's)

Source Level	DF	SS	MS	F	Significance
Total	13	0.09843			
Btwen Trt.	4	0.03444	0.00861	1.21078	0.37086
Within	9	0.06399	0.00711		

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APPENDIX
SOP LTP-48
EARTHWORM TOXICITY TESTS

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PURPOSE: The purpose of this SOP is to explain the procedure for determining the toxicity of test substances in soil to earthworms.

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Anderson, V., and R. McClean. 1974. Design of experiments. Marcel Dekker, Inc., New York, NY.

Dean-Ross, D. 1983. Methods for the assessment of the toxicity of environmental chemicals to earthworms. Reg. Toxicol. and Pharmocol. 3:48-59.

Karnak, R., and J. Hamelink. 1982. A standardized method for determining acute toxicity of chemicals to earthworms. Ecotox. Environ. Safety 6:216-222.

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Roberts, B., and H. Dorough. 1985. Hazards of chemicals to the earthworm. Environ. Toxicol. and Chem. 4:307-323.

Tektronix. 1981. Plot 50 (4050D03) Statistics: Analysis of variance. No. 061-2390-00. Tektronix, Inc., Beaverton, OR.

APPENDIX

PROCEDURE:

A. Earthworms are first purchased from the suggested vendors:

1. Eisenia foetida - Bert's Bait Farm, Irvine, KY (606) 723-5577.

2. Lumbricus terrestris - Mike's Bait Shop, Balt., MD (301) 793-0039.

B. Earthworms are stored in peat moss at 13.0 ± 0.2 C° after purchase. They are acclimatized for a minimum of two weeks prior to the commencement of tests.

C. Earthworm weighing

1. Earthworms should be weighed on the Fisher Scientific Model XT standard top-loading balance. Earthworms should be placed in a Pyrex 80 mm x 40 mm crystallizing dish.

2. The balance is to be calibrated in total accordance with SOP EMC-4. Before each individual weighing task, the balance/crystallizing dish combination should be tared to three significnat digits (0.00 g).

3. Earthworms are removed from a refrigerated storage bin 5 at a time in a beaker of distilled water. Because earthworm health and mobility are essential for survival, any earthworms that are noticeably different in color, rigidity, texture, motility or hydrophobicity are discarded. To remove any additional soil or sediment from the body surface, each earthworm is rinsed with an additional 5-10 mL of distilled water from a polyethylene squirt bottle. After rinsing, each earthworm is quickly dried on a paper towel.

4. After drying, each group of five earthworms is placed in a tared 80 mm x 40 mm crystallizing dish and is weighed to the nearest 0.1 mg. Earthworm weights are calculated using group means (n = 5). These groups are selected randomly, with each group weighing approximately the same total weight (± 10%). Before each successive weighing, the crystallizing dish is rinsed and tared. All weights should be recorded directly into the laboratory notebook.

D. Stock Preparation of Soil Media from Raw Materials

1. Earthworm substrate for experiments consists of a non-sterile artificial soil and distilled water media, based on the procedures of Neuhauser et al. (1985). The advantages of using an artificial soil mixture are ease of preparation and comparability to other data. The heterogeneous parameters of a reproducible artificial soil mixture limit the variability of the test that could occur if field soil was used. The components of the artificial soil are:

a.	Lime	1%
b.	Finely-ground sphagnum peat moss	10%
c.	Kaolinite clay	20%
d.	Fine sand	69%

All stock components are stored at room temperature in a 25 cm x 36 cm polyethylene container with lid. Each component is dated upon receipt and is marked with the company name. The shelf life for each stock component is indefinite as these are very inert substances. The shelf life of the test substance should not be greater than two years.

2. The test systems are prepared by mixing the artificial soil with either the test substance itself or a spike containing the test substance. (See SOP LTP-76 for the procedure on making a spike). Mixing is done in a Waring blender. Distilled water is slowly added and mixed until a uniform texture is established. (See Step E-2 for determining the correct amount of water to use). The test system so amended is then divided into replicates and placed into beakers. (See following steps for specific proportions). Five earthworms of comparable mass are added and each beaker is covered with gauze (cheesecloth). Target concentrations of test substance in soil are established in preliminary range-finding experiments, whereby five concentrations of test substance, including control treatments, i.e., replicates having no test substance added, are selected for further testing. See SOP LTP-76 for further explanation of a range-finding test.

E. The Actual Test

1. After the actual test concentrations have been determined from the range-finding test, the actual test design, using either E. foetida or L. terrestris, uses three replicates of five different levels of test substance, including a control.

2. When E. foetida are used as the test specimens they are placed into 400 mL beakers containig 200 g (dry weight) of artificial soil plus test substance. When L. terrestris are used, they are placed into 600 mL beakers containing 400 g (dry weight) of artifical soil plus test substance. Because water is essential to earthworm movement, respiration and reproduction, water is added to the test system. The amount of water added should be ideally 25% of the weight of the artificial soil and test substance mixture, e.g., 200 g dry weight for E. foetida x 0.25 = 50, so 50 g of water is added to 200 g of artificial soil and test substance.

3. An example of a test system for E. foetida, where a test substance itself is added to the artificial soil is shown in Table 1. "X" represents the name of the test substance.

Table 1. Components of soil spiked with "X".

Treatment	Soil wt. g	Distilled Water mL	"X" g	%"X" g/100g
Control (Beakers 1-3)	200.0	50.0	0.0	0.00
Level 1 (Beakers 4-6)	199.9	50.0	0.1	0.05
Level 2 (Beakers 7-9)	199.8	50.0	0.2	0.10
Level 3 (Beakers 10-12)	199.0	50.0	1.0	0.50
Level 4 (Beakers 13-15)	198.0	50.0	2.0	1.00

4. The five earthworms are randomly (random number generator) added to test systems. Individual test systems are randomly placed in adjacent locations inside a Precision low temperature incubator at 13.0 ± 0.2 °C. After 14 days the earthworms are weighed and examined for physical condition.

5. Data from experiments are recorded as shown in Table 2.

Table 2. Results of E. foetida exposed to "X".

Treatment/ Beaker id#	# worms beg. count	Tot. Wt. beg. g	# worms end count	Tot. Wt. end g	Mn Wt. beg g	Mn Wt. end g	Diff Wt. g
Control							
1	5	1.40	5	1.90			
2	5	1.45	5	2.07	1.37	1.86	+0.49
3	5	1.25	5	1.62			
Level 1							
4	5	1.28	5	1.71			
5	5	1.38	5	1.70	1.32	1.71	+0.39
6	5	1.30	5	1.71			
Level 2							
7	5	1.45	5	1.89			
8	5	1.56	5	1.98	1.48	1.90	+0.42
9	5	1.43	5	1.83			
Level 3							
10	5	1.48	5	1.89			
11	5	1.29	5	1.67	1.34	1.71	+0.37
12	5	1.24	5	1.56			
Level 4							
13	5	1.36	5	1.87			
14	5	1.29	5	1.52	1.35	1.72	+0.37
15	5	1.42	5	1.77			

6. The typical statistical methods used to evaluate earthworm data are:

a. Bartlett's test for homogeneous variance (Anderson and McClean, 1974)

b. Analysis of covariance (ANCOVA), for weight differences (Tektronix, 1981)

F. Artificial soil and earthworms are disposed of following the "Interim Guide for Chemical Waste Management at CRDEC". The nature of the test substance will determine the proper waste disposal method because all artificial soil components are inert.